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Dose-dependent activation and block by bisG10, a K^+ channel blocker, of mouse and frog skeletal muscle K_{ATP} channels

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Abstract **The effects of a K^+ channel blocker, bisG10, were examined on ATP-sensitive K^+ (K_{ATP}) channels in membrane patches excised from mammalian and amphibian skeletal muscle fibres using the patch-clamp technique. At micromolar concentrations, bisG10, added on the intracellular side, induced a strong, reversible, flickery block of K_{ATP} channels. BisG10, added on the extracellular side, was about 100-fold less potent at inhibiting channel activity. At 10 nM, intracellular bisG10 increased K_{ATP} channel activity. This activation was independent of the presence of internal ATP or Mg^{2+}. The inhibitory effect of bisG10 most likely arose from open-channel block whereas activation could result from more complex, indirect interactions.**

Key words: 1,10-Bis-guanidino-*n*-decane; ATP-sensitive K^+ channel; Patch clamp; Skeletal muscle

1. Introduction

ATP-sensitive potassium channels (K_{ATP}) are present at high density in skeletal muscle sarcolemma. It is thought that K_{ATP} channels, inactive in resting muscle, open during fatigue to reduce excitability and lead muscle to rest [1]. However, there exists yet no direct experimental evidence establishing this physiological role. Pharmacological tools have been widely used to study the physiological function as well as the molecular mechanisms of regulation of skeletal muscle K_{ATP} channels. Sulfonylureas, such as glibenclamide, are considered specific blockers of K_{ATP} channels [2,3]. Previous studies have shown that glibenclamide, at micromolar concentrations, produces complete inhibition of skeletal muscle K_{ATP} channels [4,5]. Classical channel blockers such as TEA or 4-AP are weak inhibitors of K_{ATP} channels that work only in the millimolar range [6,7].

A diverse family of organic compounds known as K^+ channel openers activate K_{ATP} channels. In skeletal muscle, these agents act at concentrations between 1 and 100 μM. Depending on their chemical structure, some of them activate the channel only in the presence of internal nucleotides while others increase channel activity in their absence [5,8].

In this paper, we investigate the effects on skeletal muscle K_{ATP} channels of 1,10-bis-guanidino-*n*-decane (bisG10), a drug reported to inhibit sarcoplasmic reticulum (SR) K^+ channels [9]. We find that bisG10 has opposite effects on K_{ATP} channels depending on the concentrations used. It blocks the channels at micromolar concentrations while it activates them at nanomolar concentrations.

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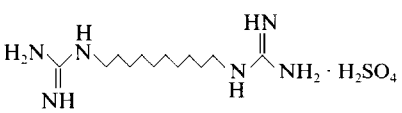
Abbreviations: bisG10, 1,10-bis-guanidino-*n*-decane; K_{ATP}, ATP-sensitive K^+ channels.

2. Materials and methods

Methods were essentially the same as in previous works [4,10]. Using the patch-clamp technique, currents through K_{ATP} channels were recorded in cell-free patches from, either enzymatically dissociated fibres from the flexor digitorum brevis and interosseal muscles of the adult mouse or sarcolemmal blebs elicited by mechanical cleavage of fibres dissected from the iliofibularis thigh muscle of the adult frog, *Rana esculenta*. Animals were killed by decapitation.

Inside/out and outside/out patches were held at 0 mV throughout in solutions designed to approximate physiological ionic gradients. The cytoplasmic face of the patch was exposed to internal solutions containing in mM: 145 K^+, 140 Cl^-, 1 EGTA, 10 HEPES, and 1 Mg^{2+} except where noted (pH 7.4) for mouse muscle and 150 K^+, 40 Cl^-, 1 EGTA, 10 PIPES, and methanesulfonate$^-$ as the remaining anions (pH 7.1) for frog muscle. The extracellular face of the patch was exposed to external solutions containing in mM: 5 K^+, 145 Na^+, 152 Cl, 1 Mg^{2+}, 2.5 Ca^{2+}, and 10 HEPES (pH 7.4) for mouse muscle and 10 K^+, 120 Na^+, 126 Cl^-, 1 Mg^{2+}, 2 Ca^{2+}, and 5 PIPES (pH 7.1) for frog muscle. ATP (potassium salt), glibenclamide (100 mM stock in dimethyl sulfoxyde), and bisG10 (1 or 2 mM stock in bath solution) were added to the bathing solution as indicated. For mouse muscle experiments, the membrane patch was exposed to the various solutions by placing the patch in the mouth of a single perfusion tube connected by valves to a number of reservoirs. For frog muscle experiments, solutions were changed by means of a rapid 'sewer pipes' device. The lower exchange speed of the former method explains the apparently slower response times of mouse K_{ATP} channels.

BisG10 (1,10-bis-guanidino-*n*-decane·H_2SO_4) was prepared from 2-methyl-2-thiopseudourea sulfate and 1,10-diaminodecane (yield: 40%) as described by Garcia and Miller [9]. The chemical structure is:



The isolated pure bisG10·H_2SO_4 gave satisfactory analytical data: IR (KBr) cm^{-1}: 3300, 3150, 3050, 1680, 1630, 1120, 1070. ^{13}C NMR (D_2O + εCF_3CO_2D), 75 MHz, δ in ppm relative to tetramethylsilane: 27.8(–CH_2–), 29.8(–CH_2–), 30.3(–CH_2–), 30.5(–CH_2–), 43.0(–CH_2–NH–), 158.5(HN=C(NH_2)–NH–). ANAL. calc. for $C_{12}H_{30}N_6O_4S$: C% = 40.66, H% = 8.53, S% = 9.05, found: C% = 40.87, H% = 8.60, S% = 8.86.

3. Results

3.1. *High-affinity blockade of mouse skeletal muscle K_{ATP} channels by intracellular bisG10*

Fig. 1A describes the effects of bisG10 added to the cytoplasmic face in an inside/out membrane patch at 0 mV with a

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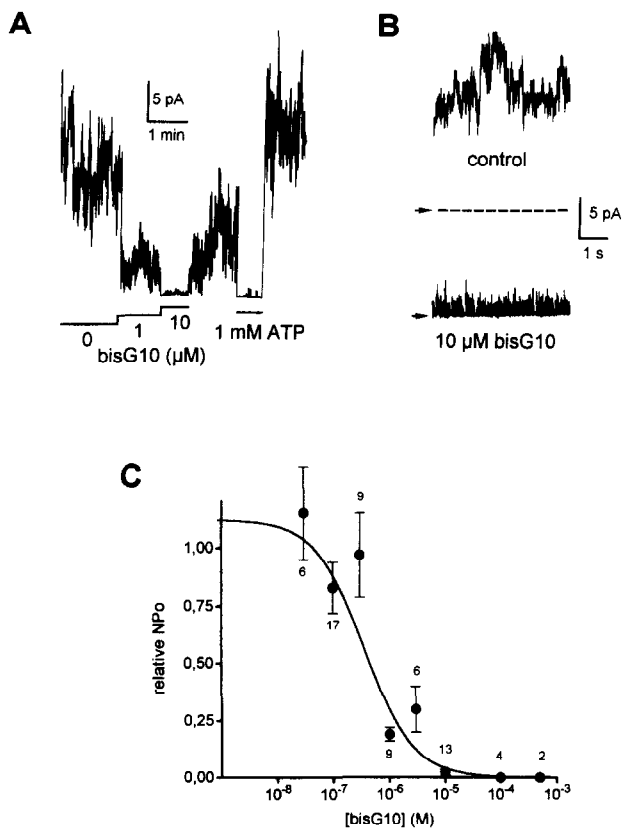

Fig. 1. Intracellular bisG10 potently blocks mouse skeletal muscle K_{ATP} channels in inside/out patches. (A) Effect of 1 μM, 10 μM bisG10 and 1 mM ATP on continuous recording of single K_{ATP} channel currents. BisG10 and ATP were added during the periods indicated by the bars. (B) Short segments of the main current trace in control and in the presence of bisG10. Arrows indicate zero current level. (C) Dose–response relationship between channel activity and internal bisG10 concentrations. Values were normalized to the value obtained in the absence of the drug. Average values of these normalized values were plotted. Bars and numbers denote number of measurements and S.E.M. value. Solid line represents the prediction from a bimolecular block model with $K_{1/2}$ = 370 nM.

physiological K^+ gradient. In the absence of ATP at the cytoplasmic face, K_{ATP} channels open spontaneously. Upon addition of increasing concentrations of bisG10 to the internal solution, channel activity decreased in a dose-dependent fashion. Mean current was reduced to 22% and 2% of control by 1 μM bisG10 and 10 μM bisG10 respectively. Short segments of the main trace displayed on an expanded time scale show that gating of the channels was altered by 10 μM bisG10 (Fig. 1B). At this concentration of blocker, channels opened and closed rapidly in a 'flickery' manner so that the open-channel current level could no longer be clearly resolved at our recording bandwidth (400 Hz). After removal of the drug, current did not return completely to its control value, reflecting more the run-down of K_{ATP} channels than the persistent action of the drug. Subsequent addition of 1 mM ATP abolished channel activity confirming that all channels present were ATP-sensitive. After application of ATP, activity increased somewhat above control as described in previous work [11].

Similar protocols were employed to test the inhibitory effect of bisG10 in a number of patches. The resulting data was used to quantify the relationship between channel activity and bisG10 concentration for concentrations above 30 nM (Fig. 1C). In spite of the dispersion of the data points due to the patch-to-patch variability of the response to bisG10, this relationship pointed to a bimolecular reaction with simple 1:1 binding of the drug with half-maximal inhibition at about 370 nM.

3.2. *Low-affinity blockade of mouse skeletal muscle K_{ATP} channels by extracellular bisG10*

The effect of an external application of bisG10 was tested in outside/out membrane patches held at 0 mV. In that configuration, K_{ATP} channels were identified by their sensitivity to glibenclamide. Fig. 2 shows that extracellular like intracellular bisG10 is able to block K_{ATP} channels. In that patch, addition of bisG10 at a concentration of 100 μM depressed channel activity by about 90%. Subsequent addition of 10 μM glibenclamide nearly blocked all channel activity. In several patches, 100 μM bisG10 and 1 mM bisG10 reduced channel activity to 33 ± 9% (n = 5) and 5 ± 4% (n = 3) of control, respectively. These observations indicate that the drug is about 100 times less active from the outside than from the inside.

In these outside/out experiments, given the physiological ionic gradients, K^+ currents through K_{ATP} channels were outwardly directed at 0 mV. Under these conditions, the drug added to the external face of the membrane moved upstream in the K^+ ion flow and one may ask whether this might be the reason for the lesser efficacy of the blocker in outside/out experiments. However, in 5 inside/out patches tested under symmetrical K^+ conditions, we observed that the block by bisG10 from the cytoplasmic side was always more efficient than the block

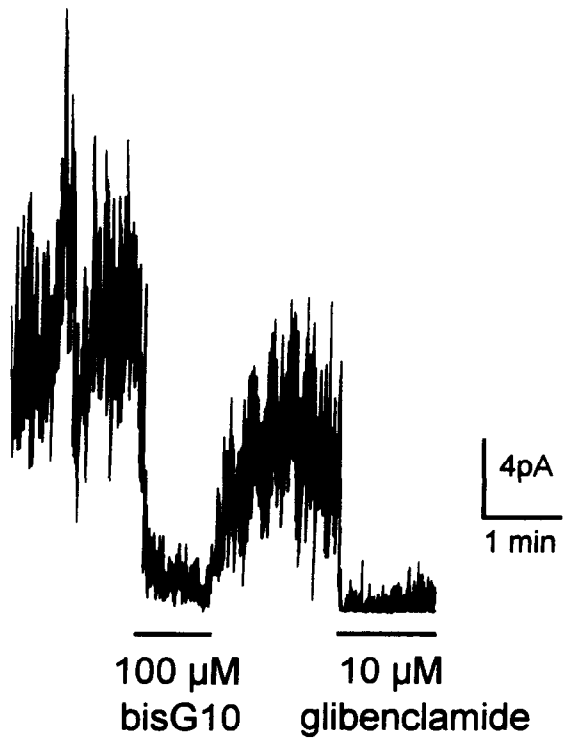

Fig. 2. Extracellular bisG10 weakly blocks mouse skeletal muscle K_{ATP} channels in outside/out patches. (A) Effect of 100 μM bisG10 and 10 μM glibenclamide on K_{ATP} channel activity. BisG10 and glibenclamide were added during the periods indicated by the bars.

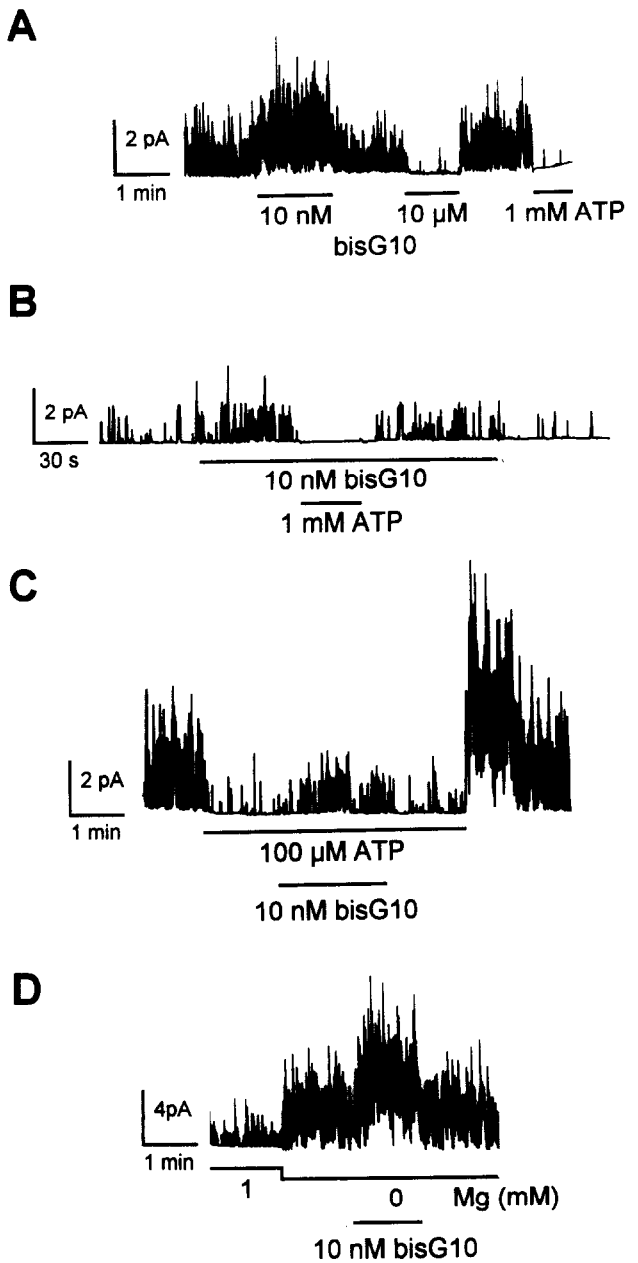

Fig. 3. Intracellular bisG10 at low concentrations activates mouse skeletal muscle K_{ATP} channels in inside/out patches. (A) Effects of 10 nM and 10 μM bisG10 in the absence of internal ATP and in the presence of 1 mM internal Mg^{2+} on continuous recording of single K_{ATP} channel currents in the same inside-out patch. (B) Effect of 1 mM internal ATP on channel activity enhanced by 10 nM bisG10 in the presence of 1 mM Mg^{2+}. (C) Effect of 10 nM bisG10 on K_{ATP} channel activity in the presence of 100 μM internal ATP and 1 mM internal Mg^{2+}. (D) Effect of 10 nM bisG10 on K_{ATP} channel activity in the absence of internal Mg^{2+}. The bars indicate the periods during which bisG10 and ATP were added and Mg^{2+} removed.

from the external side whatever the direction of the current (not shown).

3.3. *Low concentrations of intracellular bisG10 activate mouse skeletal muscle K_{ATP} channels*

Unexpectedly, we found that bisG10 behaved as an activator of K_{ATP} channels when used in the nanomolar range. Activating and blocking actions of bisG10 are shown in the same inside/out membrane patch in Fig. 3A. In this patch, addition of bisG10 at a concentration of 10 nM induced a greater than two-fold enhancement of channel activity. The activating effect was completely reversible upon washout of the drug. Subsequent addition of 10 μM bisG10 completely suppressed channel activity. This effect was reversible. Finally, ATP abolished channel activity.

On the basis of single-channel current amplitude as determined by amplitude histograms, channels opened by bisG10 could not be distinguished from K_{ATP} channels active in control conditions. Furthermore the channels activated by bisG10 were blocked by ATP as demonstrated in Fig. 3B. In that inside/out patch, channel activity which was low in control was clearly enhanced by 10 nM bisG10. In the continued presence of bisG10, addition of 1 mM ATP led to a complete inhibition of channel activity. All these effects were reversible. Activation by bisG10 was seen at 10 nM in about 70% of the patches tested (18 out of 26 patches). In those patches, 10 nM bisG10 enhanced channel opening to 218 ± 23% of control. At 30 nM bisG10, out of 6 patches tested, activation was observed in 3 patches, no effect in 1 patch and a slight inhibition in 2 patches; at 100 nM bisG10, out of 17 patches tested, activation was observed in 6 patches, no effect in 2 patches and inhibition

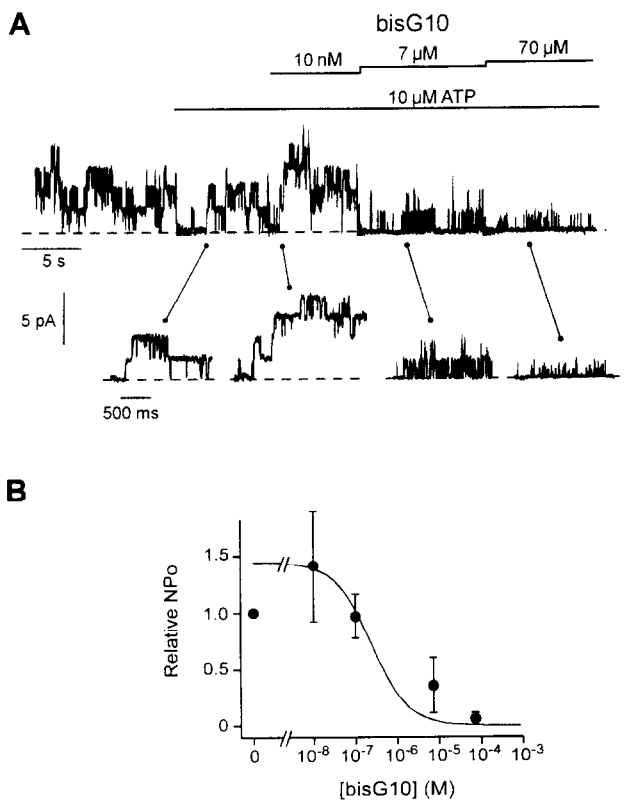

Fig. 4. Agonist/antagonist action of intracellular bisG10 on frog skeletal muscle K_{ATP} channels in inside/out patches. (A) Continuous recording of currents from a patch containing several K_{ATP} channels at different concentrations of bisG10 in the presence of a partially inhibiting dose of ATP. Designated sections of the record are displayed below on an expanded time scale. (B) Mean responses to bisG10 compiled from data obtained in 5 patches using the protocol of panel A. Symbols and bars represent mean and S.E.M. Solid line represents the prediction from a bimolecular block model with $K_{1/2}$ = 250 nM.

in 9 patches. These results can be interpreted in terms of a variable degree of overlap of the activatory and inhibitory concentration ranges of the drug in distinct patches.

It has been reported that skeletal muscle K_{ATP} channels are regulated by internal nucleotide triphosphates and diphosphates in a Mg^{2+}-dependent manner [4,10–12]. Moreover, K^+ channel openers such as cromakalim or pinacidil are thought to interact with nucleotide binding sites of the channel [5,8]. In order to determine whether bisG10 acts directly on the channel or via an interaction with nucleotide binding sites, we investigated the effects of activatory concentration of bisG10 both in the presence of ATP and in the absence of Mg^{2+} at the cytoplasmic face.

Fig. 3C indicates that an increase in activity by 10 nM bisG10 could still be obtained when channels were blocked by internal ATP. In this patch, channels which were inhibited by 100 μM ATP could be substantially and reversibly reactivated by 10 nM bisG10. Similar effects were observed in 5 patches in the presence of 100 μM internal ATP. Enhancement could not be quantified accurately in those conditions because control activity in ATP was too low.

Fig. 3D shows that bisG10 activated K_{ATP} channels in the absence of internal Mg^{2+}. As described in a previous work [11], when internal Mg^{2+} was removed, currents through mouse skeletal muscle K_{ATP} channels increased due to a parallel enhancement of channel activity and single channel current amplitude. In the absence of internal Mg^{2+}, 10 nM bisG10 augmented activity by 90%. This effect was reversible upon washout of the drug. Additional experiments indicated that in the absence of internal Mg^{2+}, channel activity was increased to 166 ± 12% of the control ($n = 4$).

3.4. *Concentration-dependent activation and blockade of frog skeletal muscle K_{ATP} channels by intracellular bisG10*

In similar conditions, internal bisG10 had very similar effects on K_{ATP} channels from amphibian and from mammalian skeletal muscle as illustrated in Fig. 4. Application of micromolar doses of bisG10 caused a profound inhibition of channel activity with a change in gating characterized by a dramatic increase in the number of brief closings. Given the precision of the experimental data, the measured dissociation constant of 250 nM for bisG10 block of amphibian channels matched well the value of 370 nM obtained for mammalian channels.

Application of 10 nM bisG10 in the presence of 10 μM ATP (a dose which produces 50% inhibition in frog [13]) resulted on average in about a 50% increase in channel activity (Fig. 4B). A concentration of 100 nM bisG10 did not significantly affect channel activity. Activation was therefore less marked in frog muscle than in mouse muscle. It was also less reproducible because observed in only about half of the patches tested.

4. Discussion

Until now, the sulfonylurea drug glibenclamide has been considered the most potent inhibitor of K_{ATP} channels [1]. In excised patches from skeletal muscle, glibenclamide produces complete inhibition of K_{ATP} channels at micromolar concentrations [4,5]. The present work demonstrates that the guanidinium derivative bisG10, primarily known as a SR K^+ channel blocker (Garcia and Miller, 1984), inhibits skeletal muscle K_{ATP} channels with an efficacy similar to that of glibenclamide. For internal bisG10, 50% block of single-channel activity requires about 300 nM while, for glibenclamide, this figure is about 200 nM in mammalian [5] and amphibian [4] skeletal muscles.

Besides K_{ATP} channels, bisG10 has been shown to block 3 other types of channels: a sarcoplasmic reticulum K^+ channel reincorporated in artificial lipid bilayers [9], the sarcoplasmic reticulum calcium release channel [14], and a K^+ channel present in blebs extruded from contracted frog muscle fibres [15]. Reported bisG10 dissociation constants for these 3 channels were respectively 50, 280, and 48 μM. Therefore, bisG10 has a much higher affinity for K_{ATP} channels but cannot be considered as specific. In particular bisG10 block was taken by Wang and Best [15] as evidence that the K^+ channels observed in membrane blebs were of SR origin in spite of differences between these channels and K^+ channels reconstituted from SR vesicles [16] and in spite of certain resemblances (conductance, inward rectification) between these channels and sarcolemmal K_{ATP} channels.

The drug bisG10 produced characteristic flickery block, i.e. rapid interruptions of the open-channel current. Similar flickery block of SR K^+ channels was induced by bis-quaternary ammonium compounds with long carbon chains in planar bilayers [17]. This flicker is thought to arise from the movement of blocking molecules into and out of the channel. The flickery block of K_{ATP} channels by bisG10 could thus reflect the presence of a binding site located within the channel conduction pathway rather than a more remote allosteric site as seems to be the case for sulfonylureas.

K_{ATP} channels were blocked asymmetrically by bisG10. The drug was found to be about 100 times more potent internally than externally. Asymmetrical block of K^+ permeation through reconstituted SR K^+ channels by bisG10 and bis-quaternary ammonium compounds has also been described [9,17]. In that case, the drugs were more effective from the cis side of the planar bilayer, presumably the cytoplasmic side, than from the trans side. However, in excised patches from skinned muscle fibre of lobster, Tang, Wang and Eisenberg [18] found that block by bis-quaternary ammonium compounds of the SR K^+ channel was more effective when the drug was added to the cytoplasmic side of the channel. The lower potency of bisG10 externally applied does not seem to entirely result from a 'clearing' of the drug by the outward K^+ current since we observed that, whatever the K^+ current direction, bisG10 always blocked K_{ATP} channels more effectively from the cytoplasmic side. It may be suggested that bisG10, when added to the external face of the membrane, binds to a site different from the binding site accessible from the cytoplasmic side.

At nanomolar concentrations, we found that bisG10 could produce significant increases in channel activity. On the basis of single-channel conductance and ATP sensitivity, the channels activated by bisG10 were identified as K_{ATP} channels. Activation of skeletal muscle K_{ATP} channels by low concentrations of bisG10 is a striking result. To our knowledge, no other drug is capable of activating K_{ATP} channels from skeletal muscle or other tissues at nanomolar concentrations. K^+ channel openers are usually effective at concentrations in the micromolar range with highest affinity in smooth muscle cells where cromakalim at concentrations of tens nanomolar induces relaxation via a postulated but controversial activation of K_{ATP} channels [1,19]. The fact that the primary action of these agents is to activate channels closed by internal ATP has led to the hypothesis that

K^+ channel openers reverse nucleotide inhibition via a competitive mechanism (e.g. [20]). That bisG10 activates K_{ATP} channels both in the absence and in the presence of internal ATP suggests that activatory action of bisG10 does not involve interaction with nucleotidic binding sites. This conclusion is strengthened by the fact that activation occurred both in the absence and in the presence of internal Mg^{2+} which is known to modulate the effects of nucleotide triphosphates as well as nucleotide diphosphates on skeletal muscle K_{ATP} channels [10–12].

The high affinity of bisG10 for K_{ATP} channels makes this compound a potentially valuable structural probe. Nonetheless, further work needs to be done in order to elucidate the mechanisms of the inhibitory and activatory actions of bisG10. Inhibition, already studied in other channel types, is probably due to open-channel block and therefore linked directly to the channel pore. However, activation by such a compound has not been described before and could arise from a more indirect interaction as suggested by our failure to observe that activation in all cases.

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